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Cutting Edge: IL-1 Receptor-Associated Kinase 4 Structures Reveal Novel Features and Multiple Conformations

Andreas Kuglstatter,1 Armando G. Villaseñor, David Shaw, Simon W. Lee, Stan Tsing, Linghao Niu, Kyung W. Song, Jim W. Barnett, and Michelle F. Browner

IL-1R-associated kinase (IRAK)4 plays a central role in innate and adaptive immunity, and is a crucial component in IL-1/TLR signaling. We have determined the crystal structures of the apo and ligand-bound forms of human IRAK4 kinase domain. These structures reveal several features that provide opportunities for the design of selective IRAK4 inhibitors. The N-terminal lobe of the IRAK4 kinase domain is structurally distinctive due to a loop insertion after an extended N-terminal helix. The gatekeeper residue is a tyrosine, a unique feature of the IRAK family. The IRAK4 structures also provide insights into the regulation of its activity. In the apo structure, two conformations coexist, differing in the relative orientation of the two kinase lobes and the position of helix C. In the presence of an ATP analog only one conformation is observed, indicating that this is the active conformation. The Journal of Immunology, 2007, 178: 2641–2645.

Interleukin-1R-associated kinase (IRAK)42 is a serine/threonine-specific protein kinase that is essential for innate (1) and adaptive (2) immune response. It plays a central role in IL-1 and TLRs signaling pathways (3, 4) and protection from bacterial infections (4). Upon IL-1 stimulation, IRAK4 and IRAK1 colocalize at the IL-1R and both kinases bind to the adaptor protein MyD88. IRAK1 is phosphorylated by IRAK4 and subsequently by autophosphorylation. Phosphorylated IRAK1 dissociates from the receptor and associates with TNFR-associated factor 6 to initiate downstream signaling cascades, resulting in activation of NF-κB, p38, and JNK mitogen-activated protein kinases. Given the central role of IRAK4 in Toll-like/IL-1R signaling and immunological protection, IRAK4 inhibitors have been implicated as valuable therapeutics in inflammatory diseases, sepsis, and autoimmune disorders (5).

IRAK4 belongs to the IRAK family of kinases, which consists of IRAK1, IRAK2, IRAK-M, and IRAK4 (3). They all contain an N-terminal death domain followed by a linker of unknown domain structure and a kinase domain. IRAK4 is the only IRAK family member that does not have a C-terminal extension. Within the kinase complement of the human genome, the IRAK4 kinase domain is relatively distinct with 38% sequence identity to the kinase domain of IRAK1 and <32% sequence identity to other human kinases. The crystal structure of the IRAK4 death domain was recently reported (6), but no structural information has been available for the kinase domain of any of the IRAK family members.

We have solved the crystal structures of IRAK4 kinase domain in its apo form, complexed with the nonhydrolysable ATP analog AMPPNP, and complexed with the pan-specific kinase inhibitor staurosporine. These structures provide valuable insight into regulation of IRAK4 kinase activity and form the framework for the design of potent and selective IRAK4 inhibitors.

Materials and Methods

Protein production

To determine the most suitable boundary of the IRAK4 kinase domain for molecular structure determination, sequential 1-aa N-terminal truncations (residues 150–165) and 3-aa C-terminal truncations (residues 417–453) were made from IRAK4 cDNA. Truncated IRAK4 constructs were generated by overlap-extension PCR in which (from the N termini) a 6-His tag, TEV protease spacer, and TEV cleavage site were encoded. PCR products were subcloned into the baculovirus transfer vector pVL1392 (BD Biosciences), and DNA sequence was verified. Correct constructs were cotransfected into Sf9 cells, and the produced virus was plaque-purified to generate clonal populations. Titered, plaque-purified virus was used to determine that a multiplicity of infection of 0.3 with a harvest time of 72 h was optimal for protein expression. An N-terminal truncation in which amino acid residues 160 through 460 of IRAK4 are expressed was one candidate identified to possess good expression properties.

For large-scale purification of IRAK4 kinase domain, pellets from 10 L of infected S9 cell were resuspended in 600 ml of lysis buffer (50 mM HEPES (pH 7.5), 300 mM NaCl, 10 mM 2-ME, 12 Complete protease inhibitor tablets (Roche Applied Science), 5 mg of DNase per 100 mg of cells). Cells were then lysed with a microfluidizer (Microfluidics) at <9,000 psi on ice. The lysate was centrifuged at 25,000 × g, and the supernatant was applied to a 50-ml Talon Superflow metal affinity column (BD Biosciences) pre-equilibrated in

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2 Abbreviation used in this paper: IRAK, IL-1R-associated kinase.
column buffer A (50 mM HEPES (pH 7.5), 300 mM NaCl, 10% (v/v) glycerol, 10 mM 2-ME, and 20 mM imidazole). The bound protein was eluted with buffer B (buffer A with 100 mM imidazole (pH 7.5)) and concentrated to 5 mg/ml. The 6-His tag was cleaved overnight at 4°C with AcTEV protease (Invitrogen Life Technologies) in 0.3% (v/v) n-octyl-β-D-glucopyranoside, <20 mM imidazole, 1× stock buffer, 1 mM DTT, and 2,000 U AcTEV. The reaction mixture was passed over a 5-ml Ni-HP column (GE Healthcare) pre-equilibrated in buffer A, and the flow-through was concentrated to 5–10 ml with an Amicon Ultra-15 concentrator (Millipore). IRAK4 kinase domain was finally purified on a Superdex 200 26/60 column (GE Healthcare) and concentrated to 13 mg/ml.

Enzymatic assay
IRAK4 kinase domain activity was measured by phosphorylation of the IRAK1 peptide substrate KKARFSRPAGGSSQSSMRV (Anaspec) using [γ-32P]ATP (Amersham). The enzyme reaction was conducted in 25 mM HEPES (pH 7.5), 2 mM MgCl2, 150 mM NaCl, 20 mM MgCl2, 0.001% Tween 20, and 0.1% bovine γ-globulin (Sigma-Aldrich). For ATP Km determinations, initial rates of enzymatic reactions were measured using 4 μM enzyme in the presence of 4.5 mM peptide substrate and 0.1–6.4 mM ATP. The inhibition potency of staurosporine was determined by measuring the activity of 2 μM IRAK4 kinase domain in the absence and presence of 10 inhibitor concentrations at 0.25 Km concentrations of both ATP and the peptide substrate.

Crystallization, data collection, and processing
The IRAK4 kinase domain at a concentration of 13 mg/ml (50 mM HEPES (pH 7.5), 10% (v/v) glycerol, 300 mM NaCl, 10 mM 2-ME, and 0.02% (v/v) octyl-β-D-glucopyranoside) was incubated for 2 h at 4°C either with 1 mM staurosporine (Sigma-Aldrich) and 5% (v/v) DMSO or with 12 mM AMPPNP (Sigma-Aldrich). The protein was crystallized by vapor diffusion in hanging drops at 20°C. A total of 0.7 μl of protein solution was mixed with 0.7 μl of 20 mM sodium malonate, 100 mM sodium acetate (pH 5.0), and 10 mM DTT resulting in a final pH of 7.0. Crystals of space group C2 (a = 147 Å, b = 139 Å, c = 89 Å, β = 124°) appeared within 3–10 days and grew to a maximum size of 500 × 500 × 200 μm³. Because IRAK4 crystals grown after incubation with AMPPNP showed no ligand density at the ATP binding site, they were soaked with saturated solution of AMPPNP for 6 h.

X-ray diffraction data of apo and AMPPNP-bound IRAK4 kinase domain was collected at the synchrotron beam line 9-1 of the Stanford Synchrotron Radiation Laboratory (Palo Alto, CA) using an ADSC Quantum 315 CCD detector. Data of the IRAK4-staurosporine complex was collected at beam line 5.0.3 of the Advanced Light Source (Berkeley, CA) using an ADSC Quantum 315 CCD detector. Data Bank accession no. 1UWH). Regions of very low sequence similarity were identified by the program XSAE (C. Broger, F. Hoffmann-La Roche, Basel, Switzerland). Molecular replacement was performed with the program Phaser (9). The search model for molecular replacement is based on the crystal structure of the proto-oncogene serine/threonine-protein kinase B-RAF (Ref. 8; Protein Data Bank accession no. 1UWH). The structure model was built with the graphics software MOLOC (11) and validated with PROCHECK (12). Illustrations of the final IRAK4 crystal structures were created using PYMOL (Delano Scientific).

Results and Discussion
Overall structure and ligand binding
We have determined the x-ray crystal structures of IRAK4 kinase domain in its nonliganded form as well as complexed with the natural microbial alkaloid staurosporine and the nonhydrolysable ATP analog AMPPNP (Table I). The IRAK4 kinase domain protein selected for crystallization is catalytically active (ATP Km = 650 μM, substrate Km = 1100 μM, kcat = 420 min⁻¹). IRAK4 kinase domain crystals contain four protein chains in the asymmetric unit. Although several structural differences are observed between the four proteins in the asymmetric unit and between the different ligand complexes at the level of individual amino acids and positioning of secondary structure elements, their overall architectures are conserved. IRAK4 kinase domain possesses the typical two-lobe structure. The N-terminal lobe consists of a five-stranded antiparallel β-sheet, helix C, and a nonprototypic N-terminal α-helix (helix B) that packs against the β-sheet (Fig. 1). In addition, a unique loop is present in the IRAK4 kinase domain between the N-terminal helix and the first β-strand, which is significantly shortened by this insertion. The C-terminal kinase lobe of IRAK4 is comprised of five larger and two shorter α-helices, one β-hairpin, the activation loop, and several additional loop structures. The N- and C-terminal loops are connected by the so-called hinge sequence, which partially defines the binding site for ATP and ATP-competitive kinase inhibitors.

Recently, three autophosphorylation sites in the activation loop of the IRAK-4 kinase domain have been identified: p-T342, p-T345, and p-S346 (13). Mutations at these positions reduce IRAK-4 kinase activity significantly. We have independently identified the same three phosphates and in electron density maps of insect cell-expressed IRAK4 kinase domain (Fig. 2A). p-T345 represents the prototypical phosphoside required for the activation of many protein kinases.

**Table 1. Statistics for x-ray data processing and model refinement**

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<tr>
<th>Data Set</th>
<th>Apo</th>
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<th>AMPPNP</th>
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<td>201C</td>
<td>201D</td>
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*a Number in parentheses are values for the highest of 10 resolution shells. Rmsd, Root mean square deviation. b Rmerge = Σh|Fh|−Σh|Fobs|/Σh|Fobs|. c Rmerge = Σh|Fhobs|−Σh|Fhcalc|/Σh|Fhcalc|. Rfree is calculated the same way using a random 5% test set of reflections.

**FIGURE 1.** Staurosporine-bound IRAK4 kinase domain. Green, Gly-rich loop; cyan, helix C; magenta, activation loop; brown, helix B; red, loop unique to IRAK4; yellow, staurosporine. Amino acids not resolved in the electron density maps are outlined as dots.
interaction contributes to the relative high ATP $K_m$ (650 μM) observed for IRAK4 kinase domain. Because the electron density of the AMPPNP phosphates is of limited quality, their interactions with the protein and solvent molecules could not be resolved unambiguously.

Unique N-terminal extension and gatekeeper residue

The primary challenge in designing therapeutic kinase inhibitors is to achieve the desired selectivity, because off-target kinase inhibition may result in adverse effects. Therefore, identification of unique features for a kinase drug target is critical for successful drug design. The N-terminal kinase lobe of IRAK4 has two defining features not seen in previously reported kinase structures: a unique N-terminal extension and tyrosine as gatekeeper residue.

In addition to the five-stranded antiparallel β-sheet and helix C, the N-terminal kinase lobe of IRAK4 contains an N-terminal extension of so far unknown function that is unique to IRAK4 as judged by a sequence alignment of 518 human kinases, including the other IRAK family members (15). It starts with an amphiphilic α-helix (helix B, aas 169–176) that makes extensive hydrophobic interactions with lipophilic side chains of amino acids forming the β-sheet (Fig. 1). This α-helix is followed by three motifs that have been described as helix-capping elements: 1) an ST motif (Ref. 16; aas 177–181), which is characterized by hydrogen bonds from the T177 backbone amide to the F180 backbone amine and from the T177 backbone carbonyl group to the D181 backbone amide; 2) an ASX motif (17, aas 181–183) defined by the hydrogen bond between the D181 side chain carbonyl group and the R183 backbone amine; and 3) a highly solvent-exposed Schellman loop (Ref. 18; aas 184–189) characterized by hydrogen bonds between the backbone amines of G188/G189 and the backbone carbonyls of P184/I185, respectively (Fig. 3). C-terminal to the IRAK4 loop continues the prototypic Gly-rich loop, which is formed by the first two strands of the five-stranded β-sheet. The Schellman loop, in particular I185, is adjacent to the ATP binding site, which makes it an ideal interaction partner for IRAK4 selective kinase inhibitors.
Immediately N-terminal to the hinge loop (aa 263–268) is the so-called gatekeeper residue, Y262 in IRAK4. The size and flexibility of the side chain of this residue generally determines whether an additional lipophilic subpocket, referred to as the “back pocket,” is present adjacent to the ATP binding site (19). The most abundant gatekeeper residue in kinases is methionine, followed by leucine, threonine, and phenylalanine. As judged by primary sequence alignments of human kinase domains, tyrosine occupies the gatekeeper residue position only in the four members of the IRAK family. The IRAK4 crystal structures reported in this study are the first structural representatives for kinases with tyrosine as a gatekeeper residue. The bulky side chain of the gatekeeper Y262 is oriented toward the conserved E233 forming a hydrogen bond with the glutamate side chain (Fig. 4). Therefore, the back pocket of IRAK4 is blocked by the gatekeeper tyrosine and not accessible for ligands. However, ligand interactions with the hydroxyl group of the gatekeeper residue Y262 should be considered in the design of IRAK family selective inhibitors.

Two kinase domain conformations and implications on IRAK4 regulation

Many kinases are activated by phosphorylation of activation loop residues, either by upstream kinases or autocatalytically. It has also been shown for several kinases that a hinge movement of helix C away from the ATP binding site is involved in switching these enzymes from their catalytically active to an inactive state (14). This process results in disruption of an essential salt bridge between an invariant lysine on strand 3 of the antiparallel β-sheet and an invariant glutamate on helix C. Several different mechanisms have been described that trigger the position of helix C and therefore the activation state of the kinase. Examples include the intermolecular interaction between helix C of cyclin-dependent kinase and cyclin (20, 21), and the intramolecular interaction between helix C of c-Src and the c-Src SH3 domain (22, 23).

The IRAK4 apo-form crystal structure shows two distinct protein conformations in the tetramer comprising the crystallographic asymmetric unit. These conformations differ significantly in their relative orientation between their N- and C-terminal lobes. The regions of the protein primarily affected by the lobe rotation of IRAK4 are the Gly-rich loop, the unique N-terminal extension, and helix C (Fig. 4). The overall effect of this rotation on helix C is a similar hinge movement as described above for other kinases. We therefore refer to the two IRAK4 kinase domain conformations as “helix C-in” and “helix C-out.” The positioning of helix C in IRAK4 has the opposite effect on the lysine-glutamate salt bridge (Fig. 4) compared with other kinases. The K213-E233 interaction in IRAK4 is only present in the helix C-out position. In the helix C-in position, the K213 side chain is disordered and the carboxyl group of E233 forms a hydrogen bond with the backbone amine of F350, which is part of the conserved DFG sequence that marks the N-terminal end of the activation loop.

In the IRAK4-AMPPNP structure, all four kinase domains present in the crystallographic asymmetric unit adopt the helix C-in conformation. As observed in the apo structure, in this conformation the K213-E233 salt bridge is not formed. Presumably, in the absence of ATP analog both kinase domain conformations coexist in solution. Binding of AMPPNP between the N- and C-terminal kinase lobes shifts this equilibrium to the helix C-in conformation. The presence of AMP-PNP at the ATP binding site was achieved by soaking AMPPNP into apo crystals, therefore the transition from the helix C-out to the helix C-in conformation can occur in the crystal lattice. The fact that AMPPNP binds only to the helix C-in conformation of IRAK4 suggests that this represents the active form of the kinase, despite the missing K213-E233 salt bridge (Fig. 4). It is possible that the presence of the hydroxyl group of the unique Y262 gatekeeper side chain affects the local hydrogen bond pattern requirements for nucleotide binding.

The mechanism of IRAK4 activation in cells is not well understood. In analogy with other kinases, it seems likely that IRAK4 phosphoryl transfer activity is regulated both by activation loop phosphorylation and by switching between the two observed (or more) kinase domain conformations (Fig. 4). In the case of IRAK4, this switch could be triggered by intramolecular interaction between kinase domain and its death domain or by intermolecular interactions with adaptor proteins like MyD88. The crystal structures of IRAK4 kinase domain presented in this study form the basis for the design of experiments that decipher these interactions, and therefore how IRAK4 mediates IL-1/TLR signaling.

Note Added in Proof. After submission of this manuscript, Wang et al. (24) published the crystal structure of IRAK4 kinase domain complexed with staurosporine and a benzimidazole inhibitor.

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Disclosures

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References

